

Sensitizing Capacity and Residual Allergenicity of Hydrolyzed Cow's Milk Formulae: Results from a Murine Model

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Key Words

Allergy · Cow's milk · Food allergy · Hydrolyzed formulae · Murine model · Sensitization

Abstract

Background: Cow's milk allergy is the most common cause of clinically relevant adverse reactions to food in infants and children. Partially and extensively hydrolyzed formulae are used for the therapy and prevention of cow's milk allergy. However, the immunogenic potency of hydrolyzed cow's milk formulae to induce and/or enhance the allergic phenotype in vivo is still under debate. The aim of this study was to assess the sensitizing capacity and residual allergenicity of various partially and extensively hydrolyzed cow's milk formulae in a murine model of cow's milk allergy. **Methods:** BALB/c mice were immunized with either a cow's milk formula or various partially and extensively hydrolyzed formulae. Immediate cutaneous hypersensitivity reactions and allergen-specific antibody production were assessed. **Results:** Although immunization with cow's milk resulted in 12/13 cases in a positive skin test response to intradermal injection of cow's milk formulae, only 1 mouse showed a positive skin test to one of the partially hydrolyzed formulae, and none showed positive reactions to other partially hydrolyzed formulae, any of the extensively hydrolyzed formulae, phosphate-buffered saline

or the amino acid formula. However, 6 of 8 mice showed positive skin tests when immunized with partially hydrolyzed formulae and with one of the extensively hydrolyzed formulae. **Conclusions:** The residual allergenic potential is markedly reduced in many hydrolyzed formulae, but most of the formulae investigated could induce an allergic immune response in BALB/c mice. Our murine model seems to be suitable to investigate the sensitizing capacity of hydrolyzed formulae and to differentiate even between extensively hydrolyzed formulae.

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Introduction

Cow's milk allergy is the most common cause of clinically relevant food allergy in infants and children. The reported prevalence in the pediatric population ranges from 0.3 to 7.5% [1–3], probably due to differences in diagnostic criteria. The treatment of choice is complete avoidance of cow's milk protein.

Besides using soy as an alternative protein source, the allergenicity of cow's milk proteins can be reduced by enzymatic hydrolysis, heat treatment and/or ultrafiltration [4]. Attempts have been made to classify products according to their degree of protein hydrolysis ('extensive' versus 'partial'), but there is no unanimous agreement on firm criteria on which to base such a classification [4].

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1018–2438/01/1254–0316\$17.50/0

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A residual allergenic potential [5–9] and sensitizing capacity [10, 11] can even be found in extensively hydrolyzed whey or casein formulae, which may lead to clinically relevant reactions in rare cases [12–15].

The second indication for administering hydrolyzed formulae to infants is allergy prevention [4]. There is still controversy as to whether partially or extensively hydrolyzed formulae should be preferred, but first studies indicate that extensively hydrolyzed formulae may be advantageous [16]. It is therefore necessary to evaluate the sensitizing capacity of commonly used or currently developed formulae with different degrees of hydrolysis. These studies are difficult to perform in humans.

The aim of this study was to develop an animal model for investigating the potential sensitizing capacity and residual allergenicity of various, differently hydrolyzed cow's milk formulae.

Materials and Methods

Immunization Protocol

Female BALB/c mice (aged 6–8 weeks) were obtained from Bornholtgard, Ry, Denmark, and maintained under pathogen-free conditions. Mice were immunized by repeated intraperitoneal injections of 10 µg of beta-lactoglobulin (BLG) (Sigma Chemie, Deisenhofen, Germany) or 1 mg of formula (Humana Milchnunion, Herford, Germany) as mentioned below, both adsorbed to 1.5 mg of Al(OH)₃ (Inject Alum, Pierce, Rockford, USA) on days 1, 14 and 21. Age-matched control animals received Al(OH)₃ in phosphate-buffered saline (PBS) in parallel. Analysis was performed on day 28 (fig. 1). The numbers of animals are given in the figures and tables. The study protocol was approved by the ethics committee responsible for studies in animals.

Investigated Formulae

As a specific positive control formula (formula 1), a nonhydrolyzed common infant cow's milk formula was used (Humana Milchnunion). As partially hydrolyzed infant formulae (formulae 2, 3 and 4), Beba HA 1 (Nestlé, Munich, Germany), Humana HA 1 and Humana 0 HA (Humana Milchnunion) were investigated. As extensively hydrolyzed formulae (formulae 5 and 6), Humana Erstnahrung and Humana VLB (Humana Milchnunion) were used. The amino acid formula Neocate (SHS, Liverpool, UK) (formula 7) served as a negative control (table 1).

Assessment of Immediate-Type Cutaneous Hypersensitivity Reactions

Intracutaneous skin testing was performed as previously described [17]. Before skin testing, mice received an intravenous bolus of Evans blue (1% w/v), the abdomen was shaved and 50 µl of test solutions were injected intradermally. At least 1.5 cm distance was kept between the injection sites in order to avoid confluence of solutions.

The following skin test solutions (50 µl per injection) were used: PBS was taken as a negative control and compound 48/80 (5 µg/ml; Sigma) was injected as a positive control. BLG (20 µg/ml), cow's milk

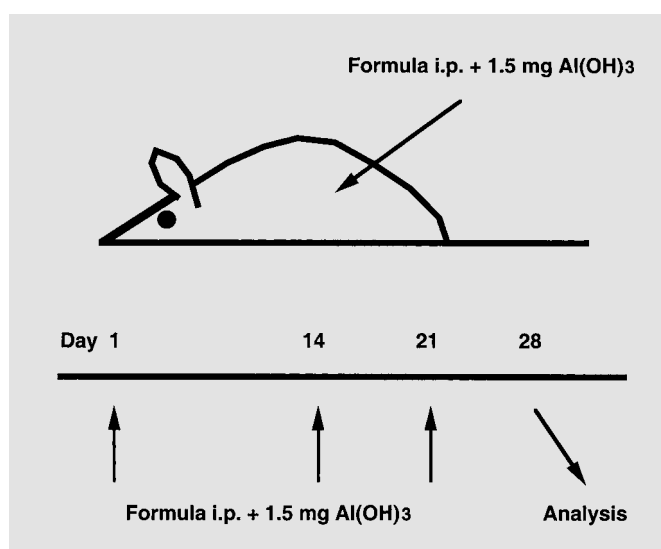


Fig. 1. Immunization protocol: female BALB/c mice received repeated intraperitoneal (i.p.) injections of 10 µg of BLG or 1 mg of formula adsorbed to 1.5 mg of Al(OH)₃ on days 1, 14 and 21. Age-matched control animals received Al(OH)₃ in parallel. Analysis was performed on day 28.

Table 1. Investigated formulae

Formula	Protein source	MW <500	MW >5,000
Formula 1 ¹	casein 40% whey 60%	0%	100%
Formula 2	whey 100%	9.1%	6.1%
Formula 3	whey 100%	12%	6%
Formula 4	whey 100%	12%	6%
Formula 5	whey 100%	55%	0%
Formula 6	casein 70% whey 30%	91.2%	0.2%
Formula 7 ²	amino acids	100%	0%

The molecular weight (MW) distribution was provided by the manufacturers.

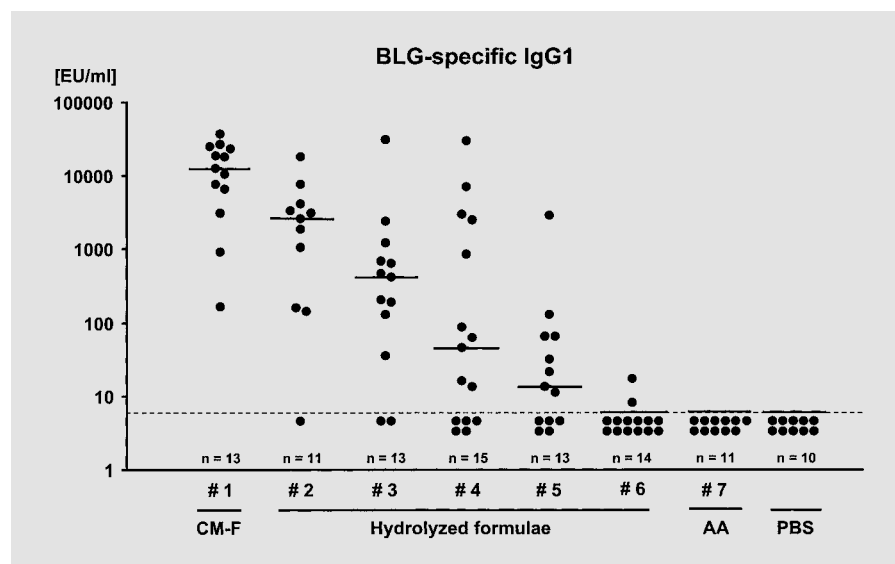
¹ Nonhydrolyzed cow's milk formula.

² Amino acid formula.

formula and partially and extensively hydrolyzed formulae 2–6 were intradermally injected at a concentration of 100 µg/ml.

Fifteen minutes later, the development of blue flare reactions was assessed on the inverted skin by an independent, blinded investigator and scored as positive if the reaction was ≥ 3 mm. Mice which did not develop positive skin test reactions to the unspecific mast cell degranulator compound 48/80 were not included in the study.

Fig. 2. Nonhydrolyzed cow's milk formula (CM-F), hydrolyzed formulae 2–6, amino acid formula (AA) or aluminium hydroxide alone (PBS) were injected intraperitoneally into BALB/c mice as described in Materials and Methods. Blood was drawn on day 28 and BLG-specific IgG1 antibody production was measured by ELISA. Every point represents one mouse. Bars represent median values. EV = Arbitrary ELISA units.



Determination of Antigen-Specific Serum IgG1 Antibody Titers
Antigen-specific serum IgG antibody titers were determined by ELISA as previously described [17]. The anti-BLG IgG1 antibody titers of the samples were related to pooled standard sera generated from BLG-sensitized BALB/c mice with a specific activity of 2,500 arbitrary ELISA units/ml.

Statistical Analysis
The Mann-Whitney U test was used to determine the level of difference between the animal groups.

Results

Sensitizing Capacity

To study the sensitizing capacity, mice received various formulae intraperitoneally; skin tests were performed with BLG and serum concentrations of BLG-specific IgG1 were determined. While none of the PBS- or amino acid formula-treated mice showed a positive intradermal skin test, a considerable number of mice were positive when immunized with partially hydrolyzed formulae and with one of the extensively hydrolyzed formulae (table 2). All animals developed positive skin tests after injection of cow's milk formula and compound 48/80; no reactions were observed after injection of PBS.

Measurement of BLG-specific IgG1 in serum revealed that the highest concentrations were observed in the cow's milk formula-immunized mice, while there was a step-wise decreased ranking towards no detectable levels in animals immunized with one of the extensively hydrolyzed formulae and the amino acid formula (fig. 2).

Table 2. Number of positive intradermal skin tests with BLG or cow's milk formula in mice after immunization with various formulae to investigate the sensitizing capacity of hydrolyzed formulae

Immunization	Skin test with BLG	Skin test with formula 1 ¹
PBS	0/8	0/8
Formula 1 ¹	7/8	8/8
Formula 2	9/10	9/10
Formula 3	6/8	6/8
Formula 4	6/8	6/8
Formula 5	6/9	6/9
Formula 6	0/9	0/9
Formula 7 ²	0/8	0/8

Cow's milk formula, hydrolyzed formulae 2–6, amino acid formula or aluminium hydroxide alone (PBS) were injected intraperitoneally into BALB/c mice as described in Materials and Methods. Skin tests were performed on day 28. PBS was used as a negative control and compound 48/80 as a positive control. Skin tests were performed with BLG (100 µg/ml) and cow's milk formula.

¹ Nonhydrolyzed cow's milk formula.

² Amino acid formula.

Table 3. Intradermal skin testing with various formulae in cow's milk-sensitized BALB/c mice to investigate the residual allergenicity of hydrolyzed formulae

Intradermal injection	PBS	Cow's milk
PBS	0/4	0/6
Formula 1 ¹	0/3	12/13
Formula 2	0/3	0/12
Formula 3	0/3	0/13
Formula 4	0/3	1/13
Formula 5	0/3	0/13
Formula 6	0/3	0/11
Formula 7 ²	0/3	0/11

Mice were injected with cow's milk formula as described in Materials and Methods. Skin tests were performed with various partially and hydrolyzed formulae as indicated.

¹ Nonhydrolyzed cow's milk formula.

² Amino acid formula.

Residual Allergenicity

To investigate the residual allergenicity of hydrolyzed formulae, nonhydrolyzed cow's milk formula was injected intraperitoneally into BALB/c mice. All but one (12/13) animal developed positive skin test reactions after intradermal injection of cow's milk formula (table 3). These reactions were associated with elevated anti-BLG IgG antibody titers (fig. 2). After intradermal injection with the various formulae, only 1 mouse (intradermally tested with the partially hydrolyzed formula 4) developed a positive skin test reaction. No skin test reactions were obtained after administration of PBS and all animals developed positive skin tests in response to the unspecific mast degranulator compound 48/80 (table 3). These results indicate that the partially and extensively hydrolyzed formulae could not elicit immediate-type cutaneous hypersensitivity reactions in cow's milk-sensitized mice.

Discussion

Our results indicate that some hydrolyzed infant formulae do show a residual allergenicity and a sensitizing capacity. Even extensively hydrolyzed formulae may inherit a small but detectable sensitizing risk, which may be of clinical relevance.

In the literature, few animal models have been used to study food allergy so far; the most widely used allergen has been hen's egg. A Dutch group intragastrically challenged ovalbumin-sensitized mice and found an important correlation between Th2 cytokines, IgE production and histamine release [18]. In a rat model, Byars and Ferraresi [19] investigated changes in the permeability of the intestinal tract after sensitization of animals with egg albumin. A cow's milk allergy model was developed by Li et al. [20], who intragastrically sensitized C3H/HeJ mice with cow's milk and proved success by determination of specific IgE to cow's milk.

When the immunogenicity of protein hydrolysis formulae was studied in a rabbit model, the authors found that animals immunized with extensively hydrolyzed proteins generated very weak immune responses [21, 22]. A group from the UK reported that milk proteins were less allergenic than ovalbumin in a rat model [23]. Animal models have also been used for studying the induction of oral tolerance [24].

Various animal species have been used for studying sensitization and allergy to food. While the mouse model is preferred by some authors [18, 20, 25], others have used rats [19, 23, 24], guinea pigs [26] or rabbits [21, 22]. In contrast, mice have been widely employed to study the regulation of IgE production under in vivo conditions. Furthermore, mice are more convenient to handle and less expensive than other species [26].

While some authors have used C3H/HeJ mice [20] or DBA/2 mice [27], we preferred BALB/c mice since systemic (intraperitoneal) injection of allergens such as pollen and animal allergens adsorbed to Al(OH)₃ resulted in a rise in total and allergen-specific antibody titers in this high-responder mouse strain [17, 18, 25]. The mice were sensitized intraperitoneally because oral sensitization has been shown to induce lower antibody titers compared to the intraperitoneal route [25]. In addition, this route allows standardization of the protein administered.

In both a murine model [27] and a rat model [28], it could be shown that oral sensitization produced specific IgE and IgG levels for casein and ovalbumin, respectively. It has previously been suggested that hydrolyzed milk formula may possess some adjuvant-like properties which may increase its ability to induce antibody production [14]. Homogenization of cow's milk is also a factor which improves the induction of sensitization in a murine model [29].

Our murine model proved to be sensitive enough to discriminate not only between partially hydrolyzed formula and extensively hydrolyzed formula, but also within

the group of extensively hydrolyzed formulae with respect to the sensitizing capacity. Additionally, more reactivity was observed to the less extensively hydrolyzed formulae, which may strengthen these results. In agreement, a Danish group found that there is a relationship between the size of peptides and the allergenicity determined by a mouse model using the passive cutaneous anaphylaxis test [26].

Many regulatory events of the murine system (including murine IgG1) parallel the function of human immune responses (e.g. human IgE). The availability of inbred mouse strains, transgenic and knockout mice and a wide variety of monoclonal antibodies make mouse models attractive for studying human diseases. Furthermore, it could be shown that passive sensitization with anti-ovalbumin IgG1 was associated with the development of skin test reactivity and increased airway reactivity after airway challenge [30].

The most important question arising from animal studies is to what extent results can be transferred to human conditions. In humans, it could be shown that

there are differences between partially and extensively hydrolyzed formulae in terms of the capacity to induce humoral responses [31]. In our murine model, a positive skin test reaction was observed in a single animal to one of the partially hydrolyzed formulae after being sensitized with cow's milk. In 41 cow's milk-allergic children, 10 positive skin prick tests (24%) were recorded when they were tested with a partially hydrolyzed formula [32].

In conclusion, our murine model seems to be suitable to investigate the sensitizing capacity of hydrolyzed formulae and to differentiate even between extensively hydrolyzed formulae.

Acknowledgements

We thank Christine Seib and Margarita Strozynski for excellent technical assistance.

This work was supported by a grant from Humana Milchunion, Herford, Germany. H.R. and U.H. are supported by grants from the 'Deutsche Forschungsgemeinschaft DFG' (Re737/4-4), the Volkswagen Foundation and by the BMBF 'Forscherguppe Allergologie'.

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